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Protein families known to be important in regulating tissue architecture and cell behavior include integrins, cadherins, Ig-CAMS, collagens, proteoglycans, tenascins, thrombospondins, matrix metalloproteases (MMPs), and many others. However, there are additional protein families that are likely to be important, but thus far have not been investigated with respect to their distribution and function in mammary tumors. One of these is a recently recognized family of transmembrane proteins containing integrin-binding domains, metalloprotease domains, and cytoplasmic domains with potential signaling capacity. This unique domain structure suggests the intriguing possibility that these molecules may play critical roles in regulating extracellular matrix and integrin function in cell adhesion and invasion. The goal of this project is to identify members of this family that are expressed in breast cancer tissue, to develop novel antibodies to them, and to determine their distribution in tumor tissue as well as in cultured breast cancer cell lines.

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FOREWORD

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R. Pytte 10/23/98

PI - Signature Date

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INTRODUCTION

The purpose of this project is provide initial data on the distribution of a novel family of extracellular proteins, termed MDCs, in breast cancer tissues. These proteins are lilely to participate in tissue remodeling because they contain domains similar to other proteins involved in cell adhesion and extracellular matrix degradation. The extracellular portion of these proteins contains a domain related to matrix-degrading metalloproteases (MMPs), and another domain similar to integrin-binding snake venom peptides (disintegrins). These domains are linked to transmembrane domains and short (15-100 amino acids) cytoplasmic domains. Some of the cytoplasmic domains contain consensus SH3-binding sequences, suggesting that MDCs can transmit signals via tyrosine kinase pathways. Sequencing of cDNAs cloned from various mammalian tissues suggests that the MDC family is very large and consists of more than 20 members that are expressed in a variety of mammalian tissues. One member of this family, termed fertilin, is expressed on the outer sperm membrane and is known to be involved in sperm-egg fusion. Fertilin has been shown to interact with the $\alpha 6\beta 1$ integrin on the egg surface. Even though only one of the known MDCs, termed metargidin, contains an RGD sequence in its disintegrin domain, it is likely that all of these proteins can interact with integrins. Several of these proteins have now been shown to have specific metalloprotease activity, and to function in crucial regulatory events, such as the activation of TNFα (TACE) or the Notch protein (Kuzbanian).

Based on the intriguing structural properties of the MDCs, we hypothesize that they may be important regulators of cell adhesion, migration, and invasion, and that specific members of this family may contribute to the invasive growth of breast cancer cells. The distribution and possible function of these proteins in normal and malignant breast tissue has not been studied. We propose to use PCR cloning in order to identify known and novel MDCs that are expressed in human and mouse mammary tumors. We will then use in situ hybridization and immunohistochemistry to determine the distribution and expression levels of individual MDCs in normal mammary gland tissue and at different stages of mammary carcinoma progression. As a mouse model of mammary tumorigenesis, we will use transgenic mice overexpressing either the polyoma middle-T or the c-neu oncogene under the control of the MMTV promoter. These mice develop invasive mammary carcinomas that are highly malignant and frequently metastasize to the lung. In addition, we will raise polyclonal and monoclonal rabbit antibodies to synthetic peptides or recombinant protein fragments based on MDC sequences. We will use these antibodies to determine the distribution of MDC proteins in human and mouse mammary tumors. We will also use antibodies to determine the subcellular distribution of MDCs in cultured cells. This will be a first step toward identifying possible interactions with integrins localized in focal contacts or at cell-cell borders. These studies will provide the foundation for further functional studies on the possible role of MDCs in breast cancer progression in vivo.

BODY:

Aim 1. To identify the MDCs expressed in human and mouse breast cancer tissue and cell lines, by using homology-based PCR cloning.

As a mouse model for breast cancer progression, we are using transgenic mouse strains that overexpress the polyoma middle T antigen, or the c-neu oncogene, under the control of the MMTV promoter. These mice were developed in the laboratory of Dr. Bill Muller, MacMaster University, Ontario, Canada. We have now established colonies of both of these mouse strains, and have collected tissues from normal mammary glands, early and late stage primary tumors, and lung metastases. In addition, we have established a series of cell lines from both tumor models. We are also routinely producing primary cell cultures from these tumors.

We have designed and synthesized oligonucleotide primers based on the conserved sequence MXHEXGHN in the metalloprotease domain, and two conserved sequences (EECDCG and ECDLXEXC) in the disintegrin domain. These primers are either degenerate (i.e., they are expected to recognize many different members of the MDC family) or specific for each known member of the family. We are currently performing RT-PCR with these primers, subcloning the amplified fragments in plasmid vectors, and sequencing inserts to confirm the identity of the products.

Aim 2. To determine the distribution of MDC mRNAs in human and mouse breast cancer tissue, by using in situ hybridization.

Work on this aim has not yet commenced. We are currently focusing on developing antibodies for immunohistochemistry. Since we have established a very successful novel method for producing rabbit monoclonal antibodies to mouse proteins, we may be able to perform all our tissue localization studies at the protein level. Thus, the much more time-consuming in situ hybridization approach me not be needed.

Aim 3. To determine the distribution of MDCs by immunohistochemistry, using antibodies raised to synthetic peptides or recombinant MDC fragments.

For these experiments, we are developing antibodies to MDCs that have been cloned and sequenced, but not characterized at the protein level. As immunogens, we are using either synthetic peptides modeled after the amino acid sequences predicted from MDC cDNA sequences, or recombinant MDC fragments expressed in mammalian cells. We are using mammalian cells transfected with either full-length MDCs or extracellular domains as a test system for antibody specificity.

We have synthesized a series of peptides based on sequences in the MDC cytoplasmic domains, the disintegrin domain, or the metalloprotease domain. Initially, we are focusing on an MDC termed metargidin, which was identified in a human breast cancer cell line, and MDC9, a widely expressed member of the family. The respective peptides are RGTKSQGPAKPPPPRKPLPAD (corresponding to a potential SH3-binding site in the cytoplasmic domain of human metargidin), and GTGWGFKNIRRGRSGGA (entire cytoplasmic domain of human MDC). We have synthesized these peptides using standard solid-phase Fmoc chemistry, using a semi-automatic apparatus that allows us to synthesize 16 peptides simultaneously.

For transfection with MDC cDNA fragments, we are using human embryonic kidney HEK-293 cells. In these cell lines, we have expressed fragments of metargidin and MDC9. Any novel MDC cDNA fragments identified in Aim 1 will also be included in these experiments. For metargidin and MDC, we are expressing either full-length cDNA or truncated cDNA lacking the transmembrane and cytoplasmic domains. In addition, we are expressing secreted forms of the disintegrin domain together with the cystein-rich domain. The cDNA constructs include a signal peptide sequence preceding the disintegrin domain, and a C-terminal hexahistidine tag to facilitate purification of the expressed protein by metal-chelate chromatography.

In order to raise monoclonal antibodies, we are immunizing rabbits with both synthetic peptides and recombinant proteins. Following several immunizations with the protein mixed with Freund's adjuvant, we obtain serum and test for presence of antibodies to the immunogen. If the response is strongly positive, we obtain the inguinal lymph nodes and spleens from the rabbit, recover the lymphocytes and freeze them in aliquots. In order to obtain rabbit monoclonal antibodies, we use a novel technique that is now firmly established in our laboratory. This technique involves fusing the lymphocytes with a rabbit plasmacytoma cell line termed 240E. This cell line was originally developed in the laboratory of Dr. Katherine Knight at Loyola University Chicago, and was optimized by several rounds of subcloning in our laboratory. We are screening hybridoma supernatants by ELISA, western blotting, and FACS. Positive hybridomas are subcloned and further characterized to confirm specificity. Specific antibodies obtained in this way will be used to detect the respective MDCs in normal mammary gland, early and late stage mammary tumors, and lung metastases.

Aim 4. To determine the subcellular distribution of MDCs in cultured cells; to determine whether MDCs are localized in focal contacts or at cell-cell borders.

Work on this aim will be initiated as soon as we obtain anti-MDC antibodies.

CONCLUSIONS:

In this first phase of our project, the work has been mainly preparatory in setting up the system that will be studied, and the tools that will be applied in the study. We have established the transgenic mouse models to be used as well as the methodology for obtaining tumor tissues and cultured cell models. We have synthesized the oligonucleotide primers and synthetic peptides that will be used in the initial phase of MDC identification. We have established RT-PCR techniques and mRNA isolation from tumor tissues. We have also established novel approaches to developing rabbit monoclonal antibodies, and this technique will be instrumental in obtaining the necessary reagents for characterizing MDCs in mouse mammary tumor tissue. Thus, in the second year of the project we plan to apply these tools in order to identify the members of the MDC family that are expressed in breast cancer tissue, and to determine their in vivo distribution with the aid of rabbit monoclonal antibodies.

REFERENCES: None

APPENDICES: None

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CAR APPROVAL LETTER

Project # 96012716

April 15, 1998

Robert Pytela, Ph.D.
Box 0854

Dept.: Medicine
Phone No.: 206-4889

Study Title: Role of Integrins in Tumor Progression in Transgenic Mice

APPROVAL NUMBER: A6169-12716-03

Approval Date: 04/07/98
Expiration Date: 04/15/99

This number is a UCSF Committee on Animal Research (CAR) number which should be used for ordering animals for this study. This number may only be used by the principal investigator and those listed as participants included in the protocol and should be referenced in any correspondence regarding this study. The committee must be notified in writing of any changes to the approved protocol including changes in personnel.

Please distribute the final approved protocol to all individual participants so that they are familiar with the procedures that have been approved. Please remember that all personnel are to be fully trained before undertaking any procedures independently.

If you have any questions, please contact the Committee on Animal Research office at (415) 476-2197, Suite 11, Laurel Heights, Box 0962, or by electronic mail at carora@itsa.ucsf.edu.

SPECIES NAME	TOTAL NUMBER APPROVED					
	Purchased			Bred		
	Category A	Category B	Category C	Category A	Category B	Category C
Mice	0	6	0	0	930	0

Michael A. Heymann, M.D., Chairman
Committee on Animal Research